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INCREASED ACTIVITY OF PROTEINASE INHIBITORS IN
DISEASE RESISTANCE TO PHYTOPHTHORA INFESTANS.

The Louisiana State University and Agricultural
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INCREASED ACTIVITY OF PROTEINASE INHIBITORS IN
DISEASE RESISTANCE TO PHYTOPHTHORA INFESTANS

A Dissertation

Submitted to the Graduate Faculty of the
Louisiana State University and
Agricultural and Mechanical College
in partial fulfillment of the
requirements for the degree of
Doctor of Philosophy

in

The Department of Plant Pathology

by
Jeng-Hsiung Peng
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This dissertation is dedicated to the author's late grandfather.

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ABSTRACT

Proteinase inhibitor activity was measured in tomato plants following infection with compatible and incompatible races of Phytophthora infestans. Sibling tomato selections TS 19, compatible with both races 0 and 1 of the fungus, and TS 33, incompatible with race 0 and compatible with race 1, were used in the study. Proteinase inhibitor activity in tomato leaf extracts was assayed spectrophotometrically for its inhibitory effect on chymotrypsin and trypsin activity.

Chymotrypsin inhibitor activity (CTIA) and trypsin inhibitor activity (TIA) increased 40 and 63%, respectively, in TS 33 plants 24 h after inoculation with race 0 (incompatible) and remained elevated during the 96 h period in which measurements were made after inoculation. A general decline in the levels of CTIA and TIA occurred in TS 19 plants following infection with race 0 (compatible). Infection of TS 19 and TS 33 with race 1 resulted in a small increase of CTIA at 24 h after inoculation followed by a decline to below initial levels. The level of TIA in the TS 33-race 1 combination followed a pattern similar to CTIA, but in the TS 19-race 1 combination the TIA level declined continually after inoculation.

Proteinase inhibitor activity increased in uninoculated upper leaves of TS 33 plants after inoculation of a single bottom leaf with the incompatible race. Inoculation with the compatible race induced only about half as much increased activity. The lowest leaf of tomato plants

was inoculated and then excised at 4, 8, 12, and 24 h after inoculation. Forty-eight h after inoculation, the upper uninoculated leaves from plants in each treatment were assayed for proteinase inhibitors. The 24 h treatment resulted in an increase of CTIA and TIA by 75 and 94%, respectively. However, near maximum responses were measured in the 4 or 8 h treatment with only slight additional increases occurring in the 12 and 24 h treatments.

A rapid increase of proteinase inhibitors in tomato plants following infection with an incompatible race of P. infestans suggests that these inhibitors play a role in the tomato plants defense mechanism to this fungus.

Comparative studies were made to determine the proteinase inhibitor activity of the two potato cultivars LaChipper, field resistant to P. infestans, and Red LaSoda, no resistance to P. infestans. Crude extracts from LaChipper tubers were found to have slightly higher levels of CTIA and TIA than extracts from Red LaSoda.

Chymotrypsin inhibitors were purified from extracts of tubers by affinity chromatography. Ninety percent of the original inhibitor activity was recovered with an 8-fold increase in activity of the inhibitors. Purified chymotrypsin inhibitor preparations were fractionated by polyacrylamide gel electrophoresis. The inhibitor preparation from LaChipper separated into eight distinct bands and that from Red LaSoda into six bands. Bands one through six from both cultivars were parallel, but bands seven and eight from LaChipper were not visible in the gels from Red LaSoda. The protein bands were cut and eluted from gel sections of an unstained gel, using a stained gel as a

marker, following electrophoresis. The protein eluted from each gel section was shown to possess both chymotrypsin and trypsin inhibitor activities.

A serological relationship between some of the inhibitor fractions separated by electrophoresis and chymotrypsin inhibitor 1 was observed. A single distinct precipitin line formed between inhibitor 1 antiserum and proteins in bands six, seven, and eight of LaChipper and band six of Red LaSoda. Precipitin lines from these inhibitor fractions formed a continuous line with each other and with the precipitin line from purified inhibitor 1 when placed in adjacent peripheral wells in double diffusion plates.

I. INCREASED PROTEINASE INHIBITOR ACTIVITY IN RESPONSE TO
INFECTION OF RESISTANT TOMATO PLANTS WITH PHYTOPHTHORA
INFESTANS

INTRODUCTION

Proteinase inhibitors have been found to be widely distributed in the plant kingdom. The first known plant proteinase inhibitor was Sbl from soybean; it was shown to be the factor in soybean flour that causes growth inhibition in animals (3, 10). Numerous proteinase inhibitors have been identified since this original work. Vogel et al. (35) lists members of Leguminosae, Solanaceae, Gramineae, Chenopodiaceae and a few microorganisms in which proteinase inhibitors are known to occur. The majority of the work on proteinase inhibitors of plant origin has been done in relation to their effect on enzyme systems of mammals. The plant proteinase inhibitors are in most cases proteins with molecular weights in the range of 5,000 to 60,000 (19, 28, 35). They have been shown in general to inhibit enzymes of animals and microorganisms that have either trypsin-like or chymotrypsin-like specificities, and there are a few cases known in which they inhibit endogenous proteolytic enzymes of the plant from which they are derived (28).

Proteolytic enzymes are produced by a large number of microorganisms, including a number of plant pathogenic fungi (4, 11, 17, 23, 25) and bacteria (14, 22, 27, 33). A role for extracellular proteolytic enzymes of plant pathogenic microorganisms in pathogenesis has been speculated by numerous workers, because proteins are known to occur in plant cell walls, middle lamellae, and plasma membranes (1, 7, 18). In fact, an increase in proteolytic enzyme activity has been shown to occur in tissues of several plants following infection by fungi (11, 16, 21, 24, 32, 34) and bacterial (5, 15, 27) pathogens.

Potato tubers are known to contain several proteolytic inhibitors (28, 35). One of the inhibitors isolated from potato tubers, chymotrypsin inhibitor 1, has been shown to occur in potato leaves (29) and to accumulate in detached potato and tomato leaves (30). More recently, Green and Ryan (8) found inhibitor 1 to be induced to accumulate throughout potato and tomato plants after mechanical wounding and after Colorado potato beetles fed on single leaves. This lead to their speculation that accumulation of proteinase inhibitors has a function in the plants protective mechanism against insects and microorganisms.

The present study was initiated to determine (i) whether the proteinase inhibitor activity is altered in tomato plants infected with Phytophthora infestans (Mont.) deBary and (ii) whether changes in the inhibitory activity could be correlated with resistance or susceptibility to the fungus.

MATERIALS AND METHODS

Sibling tomato, Lycopersicon esculentum Mill., selections TS 19 and TS 33 used in this study were derived from breeding line 386-1-5 obtained from Dr. M. E. Gallegly, West Virginia University. The two tomato races of P. infestans, isolate WV 75 (race 0) and isolate WV 33 (race 1), also were obtained from Dr. Gallegly (6). TS 19 gives a compatible (susceptible) reaction to both fungal races 0 & 1; TS 33 gives an incompatible (hypersensitive resistant) reaction to race 0 and a compatible reaction to race 1.

Plants were propagated from seed planted in vermiculite saturated with modified Hoagland's solution (13). Except when indicated, the plants were grown in a growth chamber with a day temp of 25 C and a night temp of 18 C. They were illuminated 14 h daily with 1400 ft-c (1.5×10^4 lx) of fluorescent and incandescent light.

The cultures of P. infestans were maintained on lima bean agar medium (6). Inoculum was prepared by washing sporangia from 9-day old flood-seeded lima bean agar culture plates with 10 ml of water, and diluted to 45,000 sporangia per ml of water. The sporangial suspension was incubated at 12 C for 2 h to induce zoospore liberation.

Crude proteinase inhibitor extracts were prepared by grinding tomato leaflets in a mortar with 0.2 ml of a solution containing 1 M NaCl and 1 M ascorbic acid and 3 ml of 0.05 M, pH 8.2 Tris-HCl buffer per g of leaf tissue. The homogenate was strained through six layers of cheesecloth and centrifuged at 105,651 g at 4 C for 1 h. The supernatant was retained and held at 4 C until it was assayed for

inhibitor activity.

Crude plant extracts were assayed spectrophotometrically for proteinase inhibitor activity by measuring their inhibition of the esterase activity of L-chymotrypsin (Calbiochem) and trypsin (Sigma Chem. Co.). Chymotrypsin was assayed by the method of Schwert and Takenaka (32) using tyrosine ethyl ester hydrochloride (TEE) (Mann Res. Lab.) as a substrate and determined to have a specific activity of 7.5 units per mg. Trypsin was assayed by the method of Hummel (12) using p-tosyl arginine methyl ester hydrochloride (TAME) (Mann Res. Lab.) as a substrate and determined to have a specific activity of 40 units per mg.

Assays for proteinase inhibitors were made by pre-incubating 0.05 ml of plant extracts with 0.05 ml of chymotrypsin (800 µg/ml in 0.001 M HCl) or trypsin (50 µg/ml in 0.001 M HCl) for 5 min at room temp (approx. 24 C). The assay for chymotrypsin inhibitors consisted of 2.5 ml of 0.001 M TEE in 0.05 M phosphate buffer at pH 7.0, plus 0.1 ml of pre-incubated "chymotrypsin-plant extract" in the reaction cuvette. L-tyrosine was used instead of TEE in the control cuvette. Decrease in absorbance at 240 nm was plotted for 5 min. The assay for trypsin inhibitors consisted of 2.5 ml of 0.001 M TAME in 0.04 M Tris-HCl buffer with 0.01 M CaCl_2 at pH 8.1, plus 0.1 ml of pre-incubated "trypsin-plant extract" in the reaction cuvette. Autoclaved trypsin was used in the control cuvette. Increase in absorbance at 247 nm was plotted for 3 min. Percentage reduction in proteinase activity by plant extracts was determined by comparing the rate of change of absorbance over the linear portion of the plot with control assays in which the enzymes without leaf extract were added to their respective substrates.

Data in this paper are reported as inhibitor units per mg of tissue protein (see Appendix for method of calculation). Inhibitor units were calculated by the method suggested by Vogel et al. (35) i. e., "the quantity of inhibitor that reduces the rate of conversion of substrate by 1 μ mole/min under standard conditions is equal to one international inhibitor unit (IU)." The protein content of extracts was determined by the method of Lowry et al. (20).

RESULTS

Assays were made for chymotrypsin inhibitor activity (CTIA) and trypsin inhibitor activity (TIA) in terminal leaflets from TS 19 and TS 33 plants following inoculation with races 0 and 1 of P. infestans. Tests were made with plants grown at 21 and 25 C day temp with an 18 C night temp in both cases. Results were similar, therefore only the 25 C results are reported here. CTIA increased 40% within 24 h after inoculation of TS 33 with race 0 (resistant), and the level of inhibition remained high during the 4 days after inoculation in which assays were made (Fig. 1). A slight increase of CTIA occurred at 24 h when TS 33 and TS 19 were inoculated with race 1 (susceptible reactions), followed by a decline during the next 3 days to a level below the initial activity. There was a decline from the initial CTIA in TS 19 following inoculation with race 0.

TIA increased 63% within 24 h in the TS 33-race 0 combination and it remained at about the same elevated level during the 4 days assayed (Fig. 2). A 30% increase in the TIA was observed in the TS 33-race 1 combination at 24 h followed by a decline to below the initial level during the next 3 days. A decline from the initial level of TIA was measured in TS 19 plants following inoculation with race 0 or 1.

The ability of a compatible and incompatible combination of P. infestans to induce increased levels of CTIA and TIA in upper uninoculated plant parts was tested by inoculating a single bottom leaf on individual plants and then excising at 0, 4, 8, 12, and 24 h. Terminal

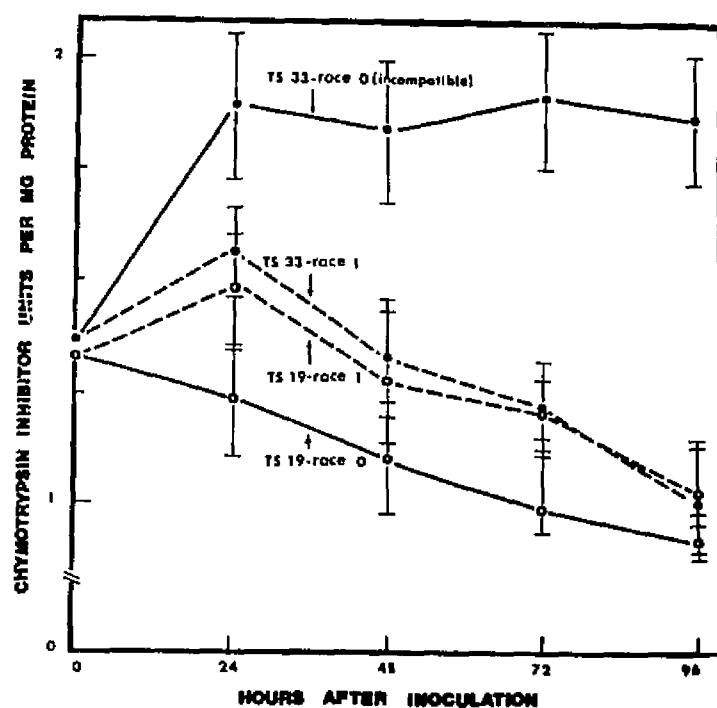


Fig. 1. The effect of inoculation of tomato plants with *Phytophthora infestans* on the stimulation of chymotrypsin inhibitor activity. TS 33 inoculated with race 0 is incompatible (resistant). All other host-fungus combinations are compatible (susceptible). Bars show standard errors of the means for four determinations of values.

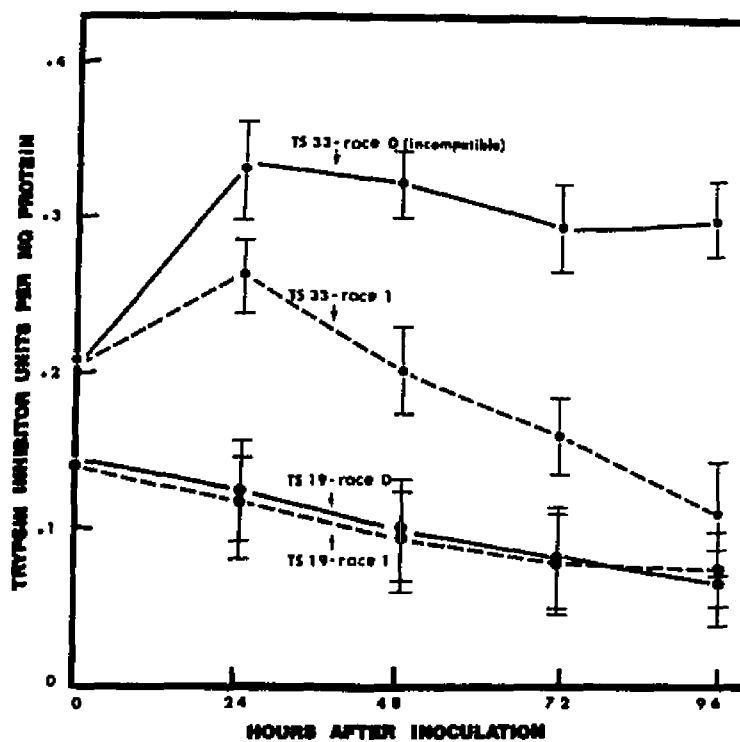


Fig. 2. The effect of inoculation of tomato plants with *Phytophthora infestans* on the stimulation of trypsin inhibitor activity. TS 33 inoculated with race 0 is incompatible (resistant). All other host-fungus combinations are compatible (susceptible). Bars show standard errors of the means for four determinations of values.

leaflets from the upper uninoculated leaves were assayed 48 h after inoculation. CTIA and TIA increased by about 72 and 95%, respectively, in the TS 33-race 0 incompatible combination (Tables 1 & 2). Near maximum accumulation of CTIA was induced even when inoculated leaves were excised 4 h after inoculation, but maximum induction of TIA did not occur until 8 h after inoculation. In the compatible combination, TS 33-race 1, CTIA and TIA were increased by about 45 and 47%, respectively (Table 1 & 2). Even though some increase in the levels of inhibition was induced within the first 4 h in the susceptible combination, maximum induction of CTIA and TIA occurred when the inoculated leaves were left attached for 8 h. Little or no further induction occurred by leaving the inoculated leaf attached for periods longer than 8 h.

In a separate experiment, tissue was cut with a cork borer from the "lesion area" and the uninvaded area of tomato leaves infected with compatible races of P. infestans, and each area was assayed for inhibitor activity. In all cases, both the CTIA and the TIA were less in the lesion area (Table 3). The CTIA was found to be higher in the uninvaded area of both TS 33 and TS 19 infected with race 1, but not with race 0, when compared with uninoculated control plants.

A single bottom leaf of TS 19 and TS 33 plants was wounded by cutting seven, 5 mm holes with a cork borer. The terminal leaflets of upper leaves were assayed to determine the effect of wounding on accumulation of CTIA. In separate experiments on plants grown at day temp of 21, 25, and 30 C, the influence of temp of CTIA was determined.

Table 1. The effect of detachment of an individual bottom leaf inoculated with *P. infestans* on the stimulation of chymotrypsin inhibitor activity in the terminal leaflets of uninoculated leaves on the same plant

| Time (h) detached after inoc. | <u>Chymotrypsin inhibitor^a</u> | | | |
|-------------------------------------|---|-------------------|----------------------------|-------------------|
| | TS 33 inoc. with race 0 | | TS 33 inoc. with race 1 | |
| | <u>IU^b</u> | <u>% increase</u> | <u>IU</u> | <u>% increase</u> |
| 0 | 1.02 a | - | 1.02 a | - |
| 4 | 1.74 b | 71 | 1.20 a | 18 |
| 8 | 1.74 b | 71 | 1.49 a | 46 |
| 12 | 1.77 b | 74 | 1.46 a | 43 |
| 24 | 1.78 b | 75 | 1.48 a | 45 |
| Not detached | 1.89 b | 85 | 1.45 a | 42 |

^aAll assays were done at the same time 48 h after inoculation of the bottom leaf.

^bMeans of four determinations; those followed by the same letter in each column do not differ significantly at the 5% level.

Table 2. The effect of detachment of an individual bottom leaf inoculated with *P. infestans* on the stimulation of trypsin inhibitor activity in the terminal leaflets of uninoculated leaves on the same plant

| Time (h) detached after inoc. | <u>Trypsin inhibitor^a</u> | | | |
|-------------------------------------|--------------------------------------|-------------------|----------------------------|-------------------|
| | TS 33 inoc. with race 0 | | TS 33 inoc. with race 1 | |
| | <u>IU^b</u> | <u>% increase</u> | <u>IU</u> | <u>% increase</u> |
| 0 | 0.18 a | - | 0.18 a | - |
| 4 | 0.23 a | 28 | 0.20 a | 11 |
| 8 | 0.34 b | 89 | 0.26 a | 44 |
| 12 | 0.33 b | 83 | 0.25 a | 39 |
| 24 | 0.35 b | 94 | 0.24 a | 33 |
| Not detached | 0.37 b | 106 | 0.24 a | 33 |

^aAll assays were done at the same time 48 h after inoculation of the bottom leaf.

^bMeans of four determinations; those followed by the same letter in each column do not differ significantly at the 5% level.

Table 3. Distribution of chymotrypsin and trypsin inhibitor activity in tomato leaves 4 days after infection with a compatible races of P. infestans

| <u>Inhibitor units/mg protein</u> | | | |
|-----------------------------------|----------------------------|----------------------------|----------------------------|
| | TS 33 inoc. with race 1 | TS 19 inoc. with race 1 | TS 19 inoc. with race 0 |
| <u>Chymotrypsin</u> | | | |
| Lesion area | 0.68 ^a | 0.71 | 0.51 |
| Uninvaded area | 2.09 | 1.53 | 0.64 |
| Healthy plants | 1.17 | 1.09 | 1.09 |
| <u>Trypsin</u> | | | |
| Lesion area | 0.11 | 0.06 | 0.05 |
| Uninvaded area | 0.17 | 0.07 | 0.07 |
| Healthy plants | 0.18 | 0.12 | 0.12 |

^aEach value average of two experiments.

Maximum accumulation of CTIA occurred in plants grown at 30 C. The level of CTIA increased with time to more than 50% above the initial 4 days after wounding (Table 4). In plants grown at 25 C, there was an increase of a little more than 20% over the same period. There was no increase in the level of CTIA at 21 C following wounding. TS 33 and TS 19 plants behaved similarly in response to wounding, however, the level of CTIA was slightly lower in TS 19 in all comparable measurements.

Table 4. The effect of daytime temp on the stimulation of chymotrypsin inhibitor activity in terminal leaflets of upper leaves following wounding^a of a single bottom leaf of tomato plants

| Hours after wounding | Chymotrypsin inhibitor units/mg protein ^b | | | | | |
|----------------------------|--|------|------|-------|------|------|
| | TS 33 | | | TS 19 | | |
| | 21C | 25C | 30C | 21C | 25C | 30C |
| 0 | 1.12 | 1.16 | 1.18 | 0.97 | 1.01 | 1.03 |
| 24 | 1.14 | 1.26 | 1.13 | 0.98 | 1.12 | 1.15 |
| 48 | 1.10 | 1.29 | 1.50 | 0.98 | 1.15 | 1.47 |
| 72 | 1.09 | 1.45 | 1.80 | 1.00 | 1.28 | 1.52 |
| 96 | 1.15 | 1.29 | 1.87 | 0.99 | 1.19 | 1.58 |

^aSeven, 5 mm holes were cut with a cork borer in a single bottom leaf.

^bData for 25 and 30 C average of three experiments and for 21 C are an average of two experiments.

DISCUSSION

The results of this study have shown a correlation between an increase of proteinase inhibitor activity following infection and resistance of tomato plants to P. infestans. The proteinase inhibitor activity of leaf extracts increased rapidly and persisted in tomato plants following infection with an incompatible race of the fungus. But, in compatible host-fungus combinations, the proteinase inhibitor activity either (i) declined following infection or (ii) increased slightly after inoculation and then declined to a level below that at the time of infection.

The exact times at which induction and subsequent accumulation of proteinase inhibitor occur were not established because measurements were not made soon enough after inoculation. By excising infected leaves at various times after inoculation, it was found that within 4 h sufficient inducer had been translocated from a single leaf inoculated with the incompatible race to elicit a near maximum CTIA response by the host. If it is assumed that infection is a prerequisite to induction and that germination and penetration is a 2 h process (26), the majority of the induction process must occur within the first 2 h after infection. The earliest assay for increased proteinase inhibitor activity was made 24 h after plants were spray inoculated, at this time the maximum increase in activity had already occurred. How soon the increase in inhibitor activity was initiated after infection and at what time the maximum level was reached was not determined.

The TS 19-race 0 combination results in a compatible reaction that is indistinguishable from TS 19- and TS 33-race 1 combinations based on disease symptoms, but there is a difference in the proteinase inhibitor response. There is a general decline of the inhibitor activity in the TS 19-race 0 combination, whereas race 1 elicits a small increase in inhibitor activity during the first 24 h after infection followed by a decline. These data suggest that the decline in the inhibitor activity is a result of invasion by the fungus as it grows in the susceptible tissue. This is substantiated by assays which show less inhibitor activity in lesions than in uninvaded portions of infected leaves or leaves from healthy plants.

The proteinase inhibitor response of the compatible combinations, TS 19- and TS 33-race 1, can be distinguished from the response of the incompatible combination, TS 33-race 0 as follows: (i) the increase of inhibitor activity in the compatible combination is less than half that in the incompatible combination, (ii) the increase of inhibitor activity is temporary in the compatible combination but persists in the incompatible combination, (iii) the increase of inhibitor activity induced in upper leaves by infection of a single bottom leaf with the compatible combination is only half as much as with the incompatible combination, and (iv) near maximal induction of CTIA occurs between 4 and 8 h in the compatible combination but between 0 and 4 h in the incompatible combination.

The increase of proteinase inhibitor activity due to fungal infection is thought not to be a wound response, because the experiments done with plants grown at 21 and

25 C gave similar results. Chymotrypsin inhibitor I, a protein proteinase inhibitor known to accumulate in tomato plants in response to wounding, accumulates optimally at 35 C, only a little at 25 C, and none at 20 C (9). Results of our assays for total CTIA in response to wounding by plants at 21, 25, and 30 C followed a similar pattern. In addition, Green and Ryan (9) showed that induction and accumulation of inhibitor I is light dependent. In our experiment, plants were kept in a dark moisture chamber for 22 h after inoculation and returned to the light chamber for only 2 h prior to the 24 h assay. The fact that maximum accumulation was measured in the 24 h assay suggests that induction and accumulation of proteinase inhibitor activity in response to infection can occur in the dark. This statement cannot be made unequivocally because of the 2 h light exposure.

The association of a rapid increase of proteinase inhibitor activity with hypersensitive resistance of tomato to P. infestans suggests a role for the increased inhibitor activity in the host's resistance mechanism. This is supported by the fact that many plant proteinase inhibitors are known to inhibit extracellular proteinases of microorganisms (28). The large increase of proteinase inhibitor activity in tomato plants in response to the incompatible race of P. infestans is suspected to be due to accumulation or to unmasking of a protein proteinase inhibitor(s), because most proteinase inhibitors from plants have been shown to be proteins (19, 28, 35). If the increase in inhibitor activity in tomato plants following infection is due to accumulation of inhibitors and not unmasking, there

remains the question as to whether the accumulated inhibitor(s) is distinct or an increase in the level of that already present. The questions raised here as to the nature of the increased inhibitor activity can be answered only by purification and identification of the inhibitor involved. It is likely that several inhibitors are involved, because a recent study showed that potato tubers had at least 13 distinct inhibitors of trypsin and chymotrypsin. (2).

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**II. COMPARISON OF CHYMOTRYPSIN INHIBITORS IN POTATO
CULTIVARS RESISTANT AND SUSCEPTIBLE TO PHYTOPHTHORA
INFESTANS**

INTRODUCTION

Various proteinase inhibitors have been identified from potatoes. Werle et al. (18) first observed that trypsin and kallikrein were inhibited by crude potato extracts. Since then two kallikrein inhibitors have been purified and characterized (12). Hochtrasser et al. (5) isolated four proteins with inhibitory activity against trypsin and some other proteinases. Balls and Ryan (1) were the first to successfully purify chymotrypsin inhibitor 1 by crystallization. Chymotrypsin inhibitor 1 was found to consist of four subunits (10). Kiyohara et al. (8) reported that potato proteinase inhibitor 1 existed as a dimer in 0.1 M acetic acid, and as a tetramer in 0.1 M NaCl. Component C of chymotrypsin inhibitor 1 is the same as proteinase inhibitor 1 in the amino acid composition, amino acid terminal residue, molecular weight and proteinase inhibitory activity (8). Iwasaki et al. (7) also isolated two other proteinase inhibitors, IIa and IIb, from potatoes, and found that the amino acid compositions and some properties were closely related to each other. Belitz (2) found as many as 13 inhibitors of trypsin and Δ -chymotrypsin to be present in potato tuber extract by polyacrylamide gel electrophoresis. It is apparent, depending on methods used and the maturity and variety of potato, that the number of proteinase inhibitors found varies greatly.

A role for proteinase inhibitors in the defense mechanism of plant tissues has been suggested (15, 17), because of their known inhibitory effect on many of the extracellular proteases of microorganisms. The purpose of the

present study was to compare proteinase inhibitor levels of the potato cultivar, Red LaSoda, which is susceptible to Phytophthora infestans with that of the cultivar, LaChipper, which has a high level of field or horizontal resistance (11).

MATERIALS AND METHODS

Chymotrypsin inhibitors were extracted from 200 g of potato tuber cut into slices with peels intact and soaked in sodium dithionite solution (7 g/l) for 2 h. The potato pieces were then homogenized in a Waring blender with about 50 ml of dithionite solution. The pulp was strained through 4 layers of cheesecloth, the expressed juice was adjusted to pH 3.0 with 6 N HCl, and centrifuged at 1000 g for 15 min at 5 C. The clear supernatant (crude extract) was used for purification of chymotrypsin inhibitors (10).

Proteinase inhibitor activity of potato tuber extracts was determined by measuring their inhibition of the esterase activity of α -chymotrypsin (Calbiochem) and trypsin (Sigma Chem. Co.). Esterase activity of chymotrypsin was determined using tyrosine ethyl ester hydrochloride (TEE) (Mann Res. Lab.) as a substrate by the method of Schwert and Takenaka (16) and that of trypsin using p-tosyl arginine methyl ester hydrochloride (TAME) (Mann Res. Lab.) as a substrate by the method of Hummel (6) with a Perkin Elmer 202 UV spectrophotometer. Determinations of enzymatic activity were made at room temp (approx. 24 C).

Assay for chymotrypsin activity. - A solution containing 2.5 ml of a 0.001 M L-tyrosine in 0.05 M phosphate buffer at pH 7.0 and 0.05 ml chymotrypsin solution (800 μ g/ml in 0.001 M HCl) were placed in the control cuvette. Two control cuvettes prepared identically were used to adjust the spectrophotometer to an absorbance reading of zero. The test cuvette contained 0.05 ml of 0.001 M TEE solution in the same phosphate buffer in place of L-tyrosine

and at zero time 0.05 ml of chymotrypsin was added to the test cuvette. Measurements of the reaction were made by plotting the decrease in absorbance at 240 nm for 5 min.

Assay for trypsin activity. - A solution containing 2.5 ml of 0.001 M TAME in 0.04 M Tris-HCl buffer with 0.01 M CaCl_2 at pH 8.1 were placed in the control cuvette and the spectrophotometer was adjusted to zero absorbance. In the control cuvette, 0.05 ml of autoclaved trypsin (50 $\mu\text{g}/\text{ml}$ in 0.001 M HCl) was added, and in the test cuvette, 0.05 ml of trypsin (50 $\mu\text{g}/\text{ml}$ in 0.001 M HCl) was added at zero time. Increase in absorbance at 247 nm was plotted for 3 min.

Assays for proteinase inhibitor activity were made by incubating 0.05 ml of inhibitor preparation with 0.05 ml of chymotrypsin or trypsin for 5 min at room temp prior to assay. Reagents used were the same as those in enzyme assay except that potato tuber extract was also added in the control cuvette and the reaction was initiated when enzyme-potato inhibitor extract was added to the test cuvette at zero time. Percentage reduction in proteinase activity by potato extracts was determined by comparing the rate of change of absorbance over the linear portion of the plot with control assays in which the enzymes without tuber extract were added to their respective substrate.

Data are expressed as inhibitor units per mg of soluble protein. One international inhibitor unit (IU) is the quantity of inhibitor that completely inhibits one unit of enzyme activity (17). Protein concentration was determined by the method of Lowry et al. (9).

Affinity chromatographic columns were prepared with 5 ml of chymotrypsin-sepharose solution (Worthington Biochem. Corp.) packed in a column (1 cm x 30 cm). Five

ml of the crude extract were applied to the column. The column was initially eluted with 66 ml of 0.05 M Tris-HCl buffer (pH 8.2). Chymotrypsin inhibitors that were bound specifically to the column were eluted with 24 ml of 6 M urea in 0.01 M HCl. Chymotrypsin inhibitors in the urea were dialyzed against several changes of the Tris-HCl buffer mentioned above. Dialyzed urea fractions containing the chymotrypsin inhibitors were pooled for gel electrophoresis.

Analytical polyacrylamide gel electrophoresis using 7% acrylamide gel was performed at 4 C according the method of Davis (3), using a Canalco disc electrophoresis apparatus. Samples containing 150-200 µg of protein in 40% sucrose solutions were placed on the top of the gel and a current of 5 ma per tube at 300 V was supplied by a Canalco power supply, model 100. Completed gels were stained for protein with 1% amido black in 7% acetic acid. Gels were destained in the same apparatus with two changes of 7% acetic acid. Percent inhibition of chymotrypsin activity of the protein in each band was determined by slicing sections from an unstained gel, using the stained gel as a marker, eluting overnight at 4 C with 0.3 ml of 0.05 M Tris-HCl buffer, pH 8.2 and assaying as described above.

Ouchterlony gel diffusion assays (13) were performed to test the immunological relationship of the standard chymotrypsin inhibitor 1 with chymotrypsin inhibitors purified in this study. Standard potato chymotrypsin inhibitor 1 and its antiserum were supplied by Dr. C. A. Ryan, Department of Agriculture Chemistry, Washington State university. The medium was prepared using 2% Noble agar (Difco) in 0.1 M sodium barbital, 0.9% NaCl, pH 8.2 and 1:10,000 thimerosal (14).

RESULTS

Crude extracts were assayed for chymotrypsin and trypsin inhibitor activities. Chymotrypsin and trypsin were inhibited greater by extracts from potato tubers of LaChipper than in those from Red LaSoda (Table 1). The chymotrypsin inhibitor activity (CTIA) of LaChipper and Red LaSoda was 2.28 and 2.0 units/mg, respectively. Whereas, trypsin inhibitor activity (TIA) of LaChipper and Red LaSoda was 0.23 and 0.18 units/mg, respectively.

Purification of chymotrypsin inhibitors. - Five ml of crude extract (clear supernatant) of LaChipper and Red LaSoda were applied to chymotrypsin-sepharose affinity chromatographic column. The second 280 nm absorbing peak eluted from the column by 6 M urea contained most of the CTIA (Fig. 1). Ninety percent of the CTIA was recovered from this single step method of purification with an 8-fold concentration of the inhibitory activity (Table 2).

Polyacrylamide gel electrophoresis of chymotrypsin inhibitors. - The purified preparation of each cultivar from affinity chromatography was placed on the cathode end of a gel column for fractionation by electrophoresis. The disc gels of purified chymotrypsin inhibitors from LaChipper and Red LaSoda show that LaChipper has eight electrophoretically distinct bands and Red LaSoda has six (Fig. 2). Protein bands one through six from both cultivars are parallel, but bands seven and eight occur only in LaChipper.

CTIA of individual protein bands from gel electrophoresis. - Disc gels were cut into slices with a razor

Table 1. Chymotrypsin and trypsin inhibitor activities of potato tubers

| Proteinase inhibitor activity | <u>Inhibitor units/mg protein</u> | |
|-------------------------------------|-----------------------------------|------------|
| | LaChipper | Red LaSoda |
| CTIA | 2.28 ^a | 2.00 |
| TIA | 0.23 | 0.18 |

^aEach value is an average of four replicates. The difference in inhibitor activity between cultivars is not statistically significant.

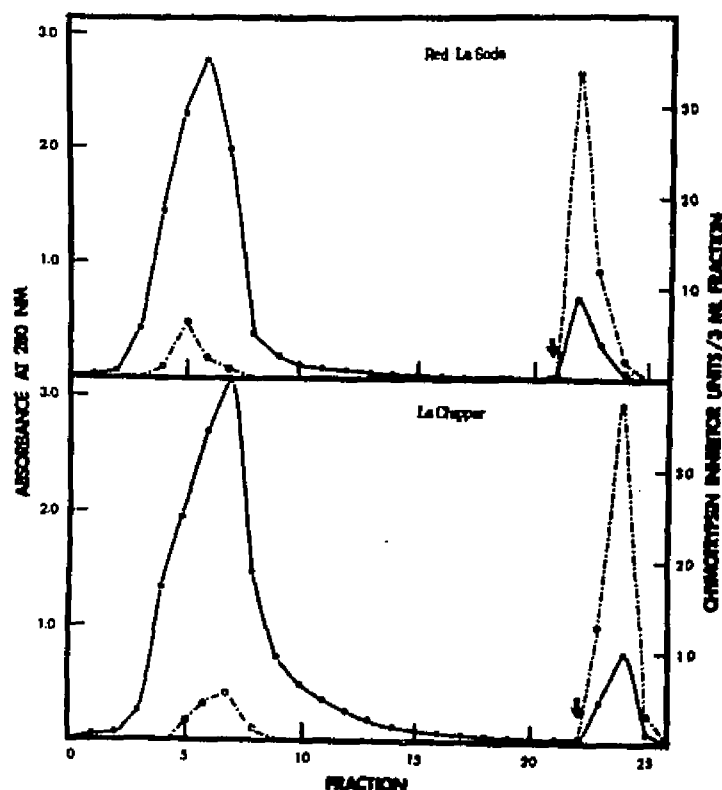


Fig. 1. Elution profile of potato chymotrypsin inhibitors extract of LaChipper and Red LaSoda on a chymotrypsin-sepharose affinity chromatographic column. Chymotrypsin inhibitor activity is expressed as units per 3 ml fraction. Symbol: $\bullet\text{---}\bullet\text{---}\bullet$, absorbance at 280 nm; $\circ\text{---}\circ\text{---}\circ$, chymotrypsin inhibitor activity. Columns were eluted with 66 ml of 0.05 M Tris-HCl buffer at pH 8.2, and then with 24 ml of 6 M urea in 0.01 M HCl. The arrow indicates the addition of urea.

Table 2. Purification of chymotrypsin inhibitors from potato tuber extracts by affinity chromatography using a chymotrypsin-sepharose column

| | Total inhibitor activity ^a | Percent yield | Specific activity ^b | Purification |
|----------------|---|------------------|-----------------------------------|--------------|
| Crude | | | | |
| LaChipper | 57.5 | 100 | 2.28 | 1 |
| Red LaSoda | 52.5 | 100 | 2.00 | 1 |
| Chromatography | | | | |
| LaChipper | 51.0 | 90 | 18.3 | 8.0 |
| Red LaSoda | 47.5 | 90 | 17.0 | 8.5 |

^aChymotrypsin inhibitor units per 5 ml crude tuber extract.

^bInhibitor units per mg tuber protein.

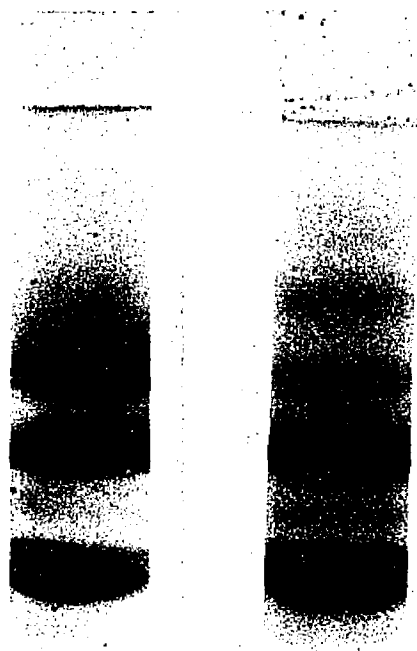


Fig. 2. Polyacrylamide disc gels after electrophoresis of the chymotrypsin inhibitors purified by affinity chromatography. LaChipper on the right and Red LaSoda on the left. Electrophoresis was performed in Tris-glycine buffer at pH 9.5 for 50 min and stained with 1% amido black.

blade and inhibitor proteins were eluted with 0.05 M Tris-HCl buffer at pH 8.2. The results of this experiment are shown in Table 3. Proteins isolated from corresponding bands one through six from the disc gels from each of the cultivars possessed similar levels of CTIA. The proteins eluted from bands seven and eight from LaChipper possessed strong CTIA, but eluates from corresponding gel sections from Red LaSoda inhibited chymotrypsin only slightly. Even though it is not shown in Table 3, all of the proteins isolated were checked and found to inhibit trypsin as well as chymotrypsin.

Immunological properties of chymotrypsin inhibitors.-

Proteinase inhibitors separated by disc gel electrophoresis and eluted from sections sliced from the gel after electrophoresis were used to test their immunological relationship with chymotrypsin inhibitor 1. No precipitate formed between chymotrypsin inhibitor 1 antiserum and the eluates from bands one through five from the gel columns. A precipitin line did occur between the proteins eluted from gel sections comparable to bands six, seven, and eight of LaChipper and chymotrypsin inhibitor 1 antiserum (Fig. 3). The precipitin lines from the tuber inhibitors connected with the lines between purified chymotrypsin inhibitor 1 and its antiserum, which indicated their serological identity. The protein from gel section corresponding to band six of Red LaSoda also gave a positive precipitin reaction with chymotrypsin inhibitor 1 antiserum.

Table 3. Comparison of chymotrypsin inhibition of inhibitor proteins eluted from electrophoresis gel columns

| Gel Fractions ^b | Percent inhibition of chymotrypsin ^a | |
|----------------------------|---|------------|
| | LaChipper | Red LaSoda |
| 1 | 4.3 | 5.8 |
| 2 | 5.6 | 7.1 |
| 3 | 14.3 | 17.6 |
| 4 | 13.6 | 11.8 |
| 5 | 13.0 | 10.4 |
| 6 | 19.8 | 24.5 |
| 7 | 13.6 | 3.8 |
| 8 | 22.8 | 2.2 |

^aAssays contained 0.3 units of chymotrypsin, the uninhibited rate results in an absorbance change of 0.18 in the 5 min assay.

^bProtein gel sections corresponding to bands were sliced and eluted with 0.3 ml of 0.05 M Tris-HCl buffer at pH 8.2 overnight at 4 C.

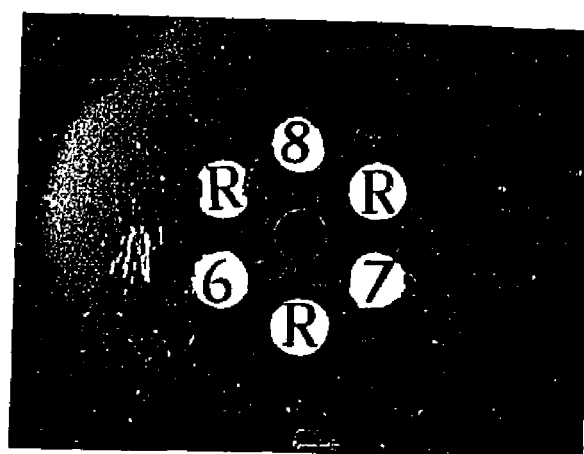


Fig. 3. Ouchterlony double diffusion test with chymotrypsin inhibitor proteins corresponding to bands 6, 7, and 8 from gel electrophoresis columns of LaChipper potatoes and purified chymotrypsin inhibitor 1 (R) in the peripheral wells and chymotrypsin inhibitor 1 antiserum in the center well.

DISCUSSION

The field resistant cultivar, LaChipper, was found to have a slightly higher level of both CTIA and TIA than the susceptible cultivar Red LaSoda. Gel electrophoresis of inhibitors purified by affinity chromatography from tuber extracts showed LaChipper to have two proteins in addition to the six that it had in common with Red LaSoda. Proteins eluted from gel sections corresponding to the two additional bands exhibited strong chymotrypsin inhibition, while eluates from corresponding sections cut from disc gels of Red LaSoda exhibited weak inhibition. The low level of inhibitor measured at position seven and eight in the Red LaSoda gel may be either low levels of the same inhibitor fraction as that from LaChipper or it may be contamination from the other inhibitor fractions.

Inhibition of trypsin by inhibitors purified by chymotrypsin-sepharose affinity chromatography was not unexpected because many proteinase inhibitors inhibit both chymotrypsin and trypsin activities (2, 5).

CTIA of isolated inhibitor protein from the two potato cultivars were compared. Bands one through six in the electrophoretic columns were identical in position and proteins isolated from them were similar in the CTIA from each cultivar. Proteins eluted from gel sections corresponding to bands seven and eight, that occurred only in extracts from LaChipper, strongly inhibited chymotrypsin and apparently are responsible for the higher total CTIA exhibited by LaChipper extracts. Since the protein in each of these gel sections reacts serologically with chymotrypsin

inhibitor 1 antiserum they are assumed to be components of that inhibitor. Inhibitor 1 has been shown to dissociate into four subunits (protomers) that are serologically identical, but with different inhibitory, electrophoretic, and chromatographic properties (10). Therefore, it is not surprising that more than one inhibitor protein from the gel columns reacts positively with inhibitor 1 antiserum.

As was pointed out earlier, numerous proteinase inhibitors have been found in potato tubers. No definite function has been found for these inhibitors, but it has been hypothesized that they serve as storage proteins (15) and that they have a role in defense against microorganisms (17). The association of higher levels of proteinase inhibitor activity with field resistant potatoes in the present study is circumstantial evidence that supports the suggested role of inhibitors in the plants defense against microorganisms.

Recently it has been shown that proteinase inhibitor activity is markedly increased in tomato plants in response to infection with an incompatible race of Phytophthora infestans (Section 1 of this Dissertation). In light of this, it seems obvious that these studies on resistant and susceptible potato tubers should be extended to include proteinase inhibitor measurements following inoculation with P. infestans to determine whether or not there is an induction of inhibitors in infected tubers that can be associated with resistance.

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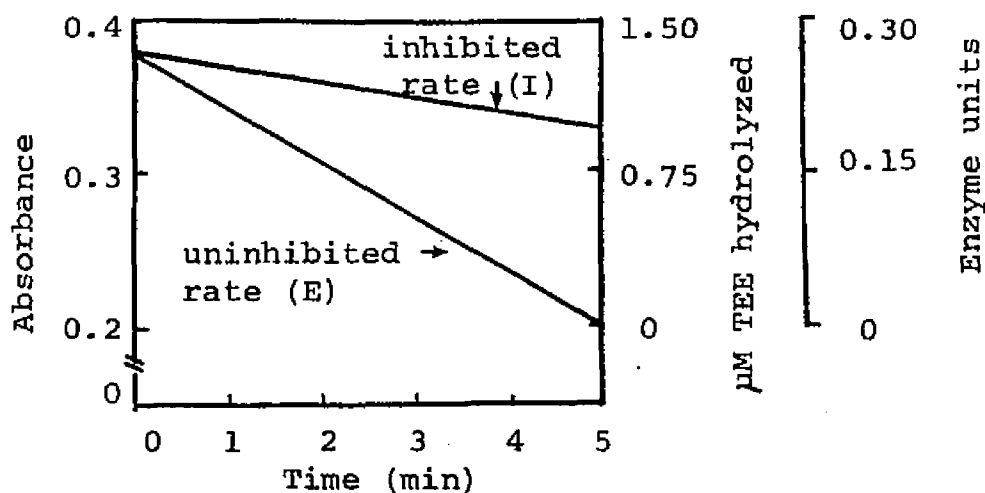
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APPENDIX

Formula for Calculation of Inhibitor Units:

$$\text{IU/mg protein} = \left(\frac{\Delta A_E - \Delta A_I}{\Delta A_E} \right) \times U \div 0.05 \div \text{mg tissue protein per ml}$$

Chymotrypsin Inhibitor Calculation:



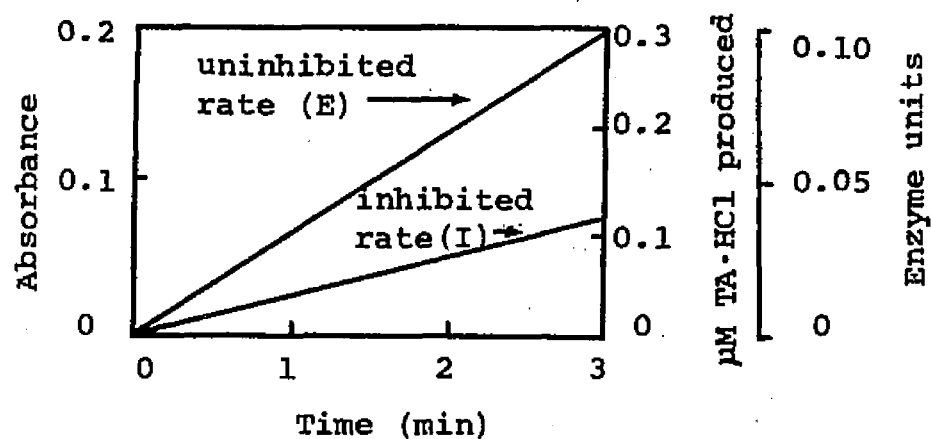
Assay for chymotrypsin inhibitor units using 40 μg chymotrypsin and 2.5 μM TEE per 2.6 ml cuvette contents.

Enzyme units = 0.3

Example calculation for chymotrypsin IU : $\Delta A_E = 0.18$; $\Delta A_I = 0.05$; $\mu = 0.3$; and protein = 3.2 mg/ml

$$\begin{aligned} \text{Chymotrypsin IU/mg protein} &= \left(\frac{0.18 - 0.05}{0.18} \right) \times 0.3 \div 0.05 \\ &\quad \div 3.2 \\ &= 1.34 \end{aligned}$$

Trypsin Inhibitor Calculation:



Assay for trypsin inhibitor units using 2.5 μg trypsin and 2.5 μM TAME per 2.6 ml cuvette contents.
Enzyme unit = 0.1

Example calculation for trypsin IU : $\Delta A_E = 0.2$;
 $\Delta A_I = 0.085$; $\mu = 0.1$ and protein = 4.7 mg/ml

$$\begin{aligned} \text{Trypsin IU/mg protein} &= \left(\frac{0.2 - 0.085}{0.2} \right) \times 0.1 \div 0.05 + 4.7 \\ &= 0.24 \end{aligned}$$

VITA

Jeng-Hsiung Peng was born on May 23, 1940 in Hsinchu, Taiwan, China. He graduated from Chung-Hsing University, Taiwan and received a bachelor of Science degree in June, 1964. He began his graduate work in the Department of Plant Pathology, Louisiana State University in September, 1968 and received a Master of Science degree in January, 1971. He is a candidate for the doctor of Philosophy degree in plant pathology in December, 1974.

EXAMINATION AND THESIS REPORT

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Major Field: Plant Pathology

Title of Thesis: Increased Activity of Proteinase Inhibitors in Disease
Resistance to Phytophthora infestans

Approved:

Lowell L. Black

Major Professor and Chairman

James G. Traenkle

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Date of Examination:

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